LYMPHATIC PUMP TREATMENT ENHANCES PULMONARY IMMUNITY AND INHIBITS SOLID TUMOR FORMATION IN THE LUNG

THESIS

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NK cells	3.6 ± 1.0	3.5 ± 0.8	7.9 ± 2.4*
B cells	2.8 ± 1.3	3.2 ± 1.0	6.8 ± 3.2*
$CD4^+$ T cells	1.5 ± 0.4	2.0 ± 0.6	3.0 ± 0.8*
$CD8^+$ T cells	3.1 ± 0.8	3.8 ± 1.4	7.8 ± 2.3*

Table 1. LPT increases leukocytes in the lungs of rats with pulmonary tumors. Rats were intravenously injected with 1 x 10^6 tumor cells in log phase. For days 1-7, the control group received no treatment or anesthesia, the sham group received 4 min of light touch (under anesthesia), and the LPT group received 4 min of LPT (under anesthesia). On day 8, rats were euthanized and the lungs removed to enumerate leukocyte populations. Data are means ± SEM total lung leukocytes x 1 x 10^5 (N=10 rats per group). * denotes $P \leq 0.05$ compared to control and sham

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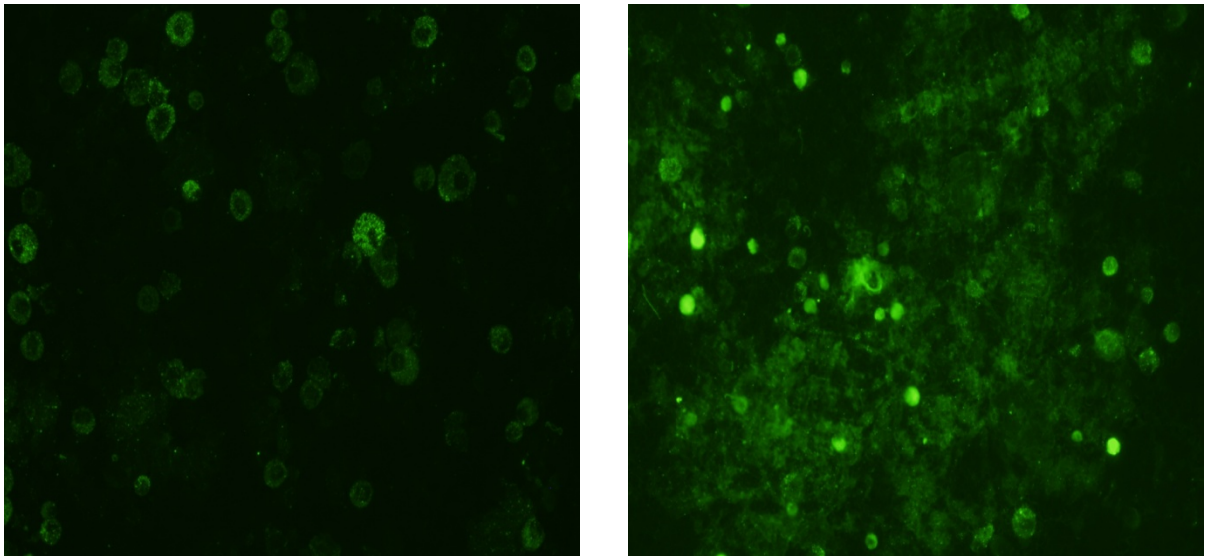
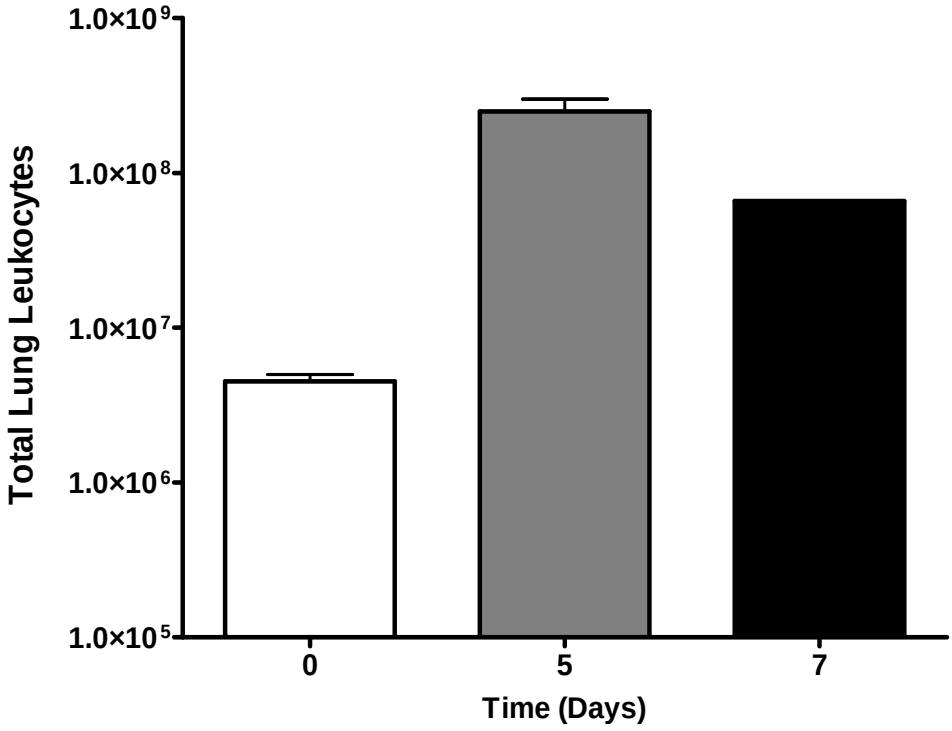


A **B**

Figure 1. I.V injection of fluorescently labeled tumor cells results in pulmonary metastasis by day 5. On day 0, rats were intravenously injected with 10 x 10^6 CFSE-labeled MADB106 tumor cells in 1ml sterile saline. On day 5 (A) and 7 (B), lungs were homogenized and analyzed for the presence of CFSE-labeled tumors by fluorescence microscopy.



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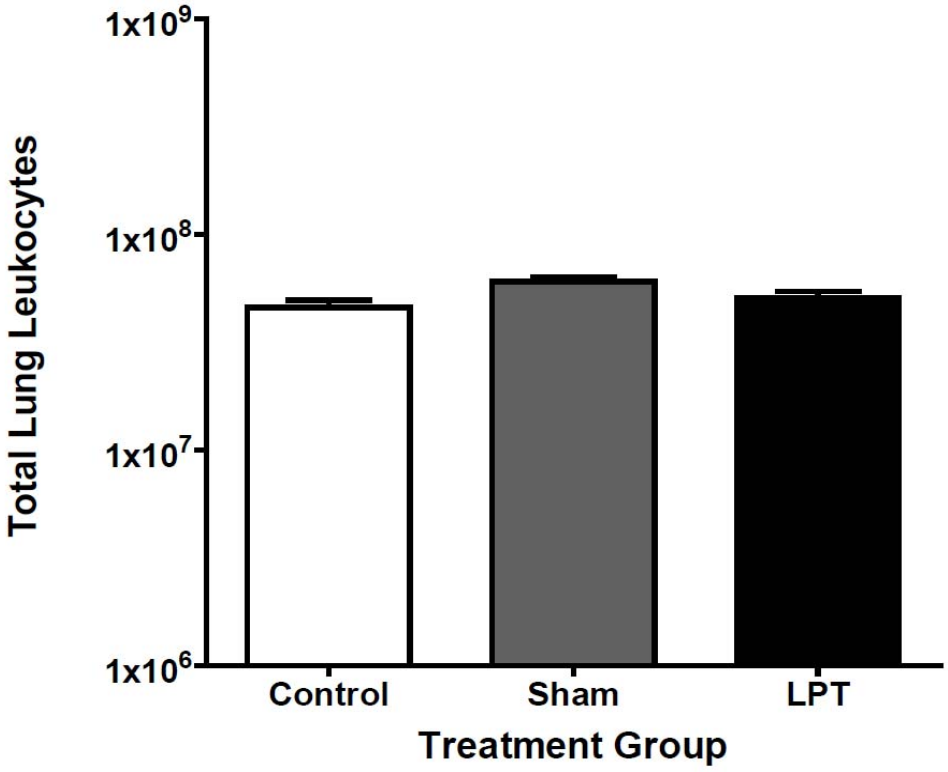

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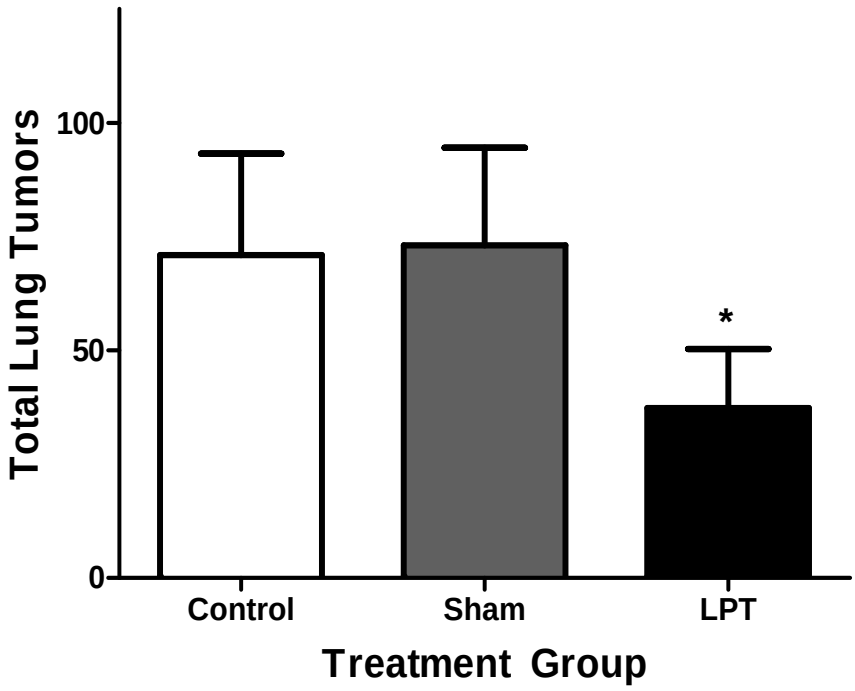

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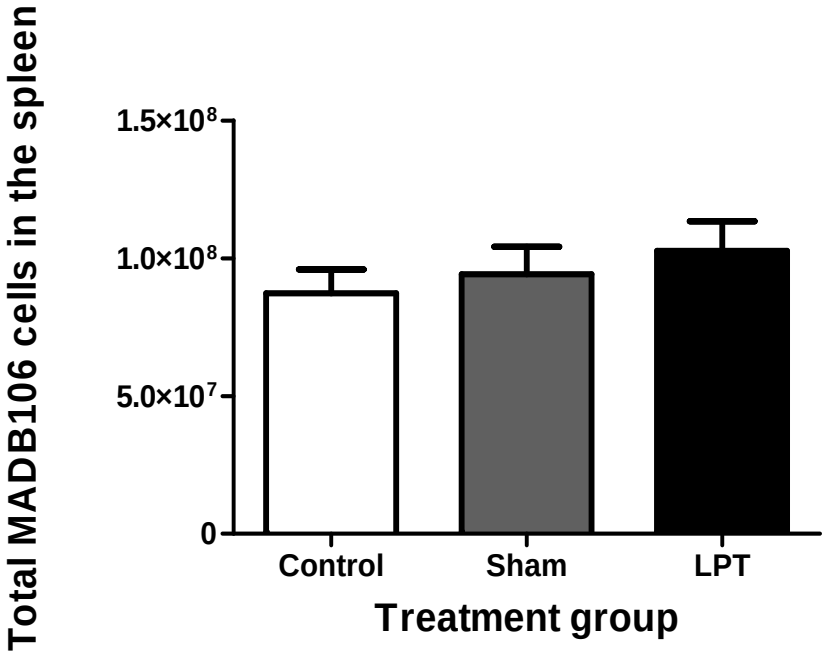

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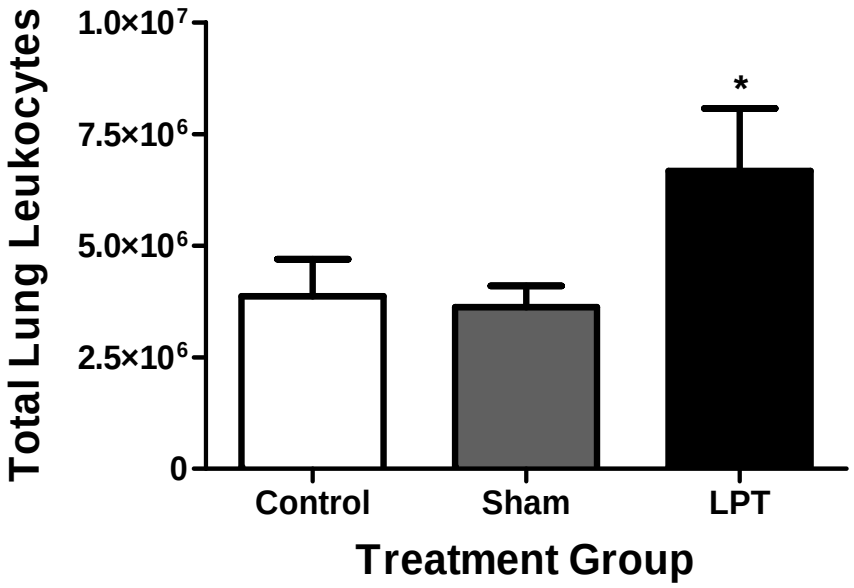

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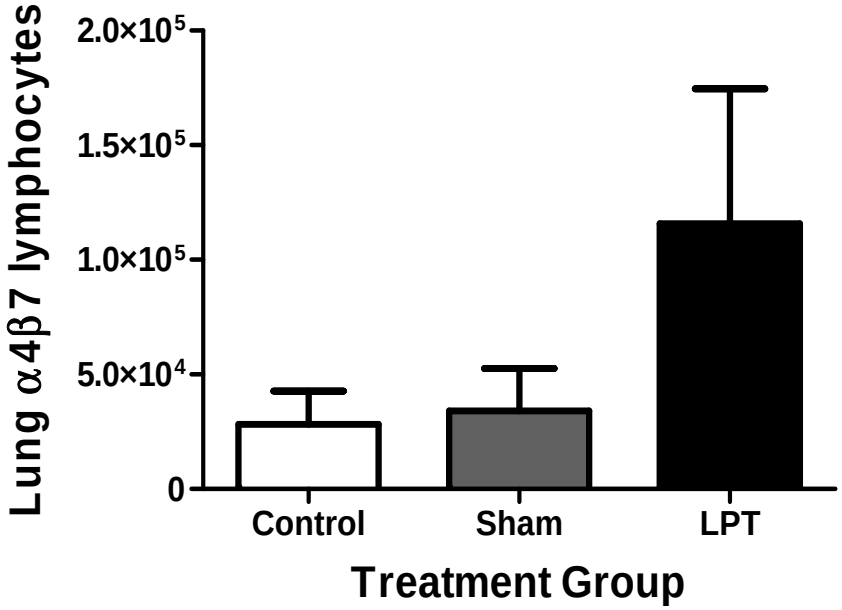

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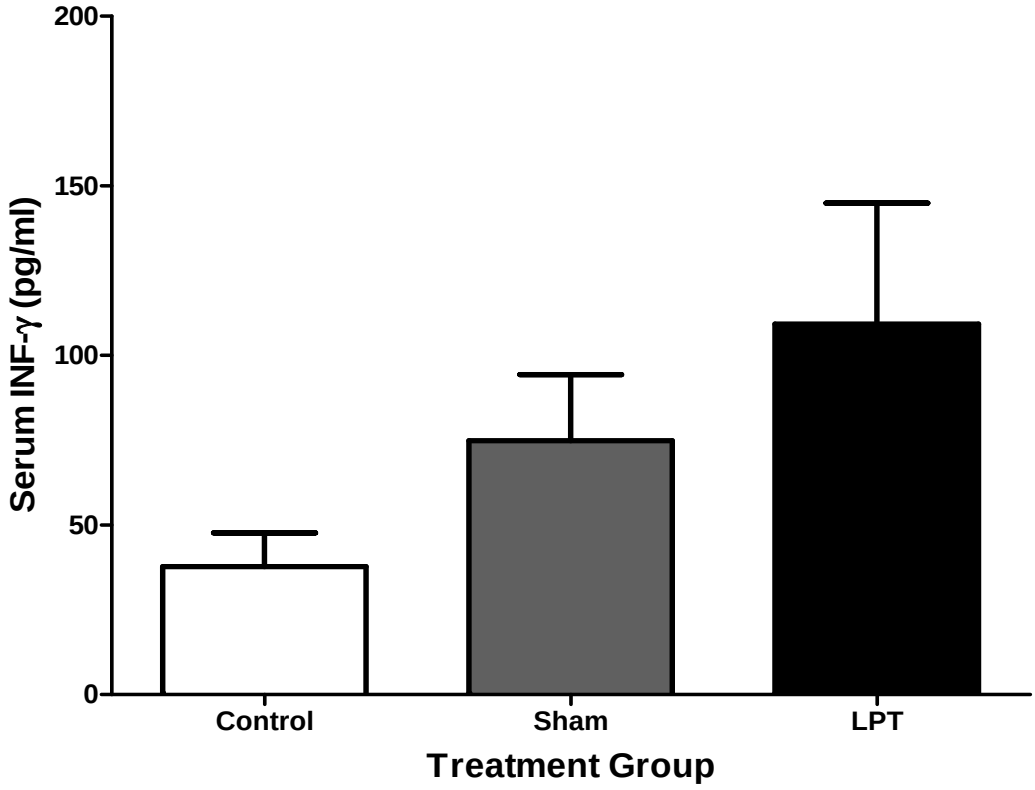

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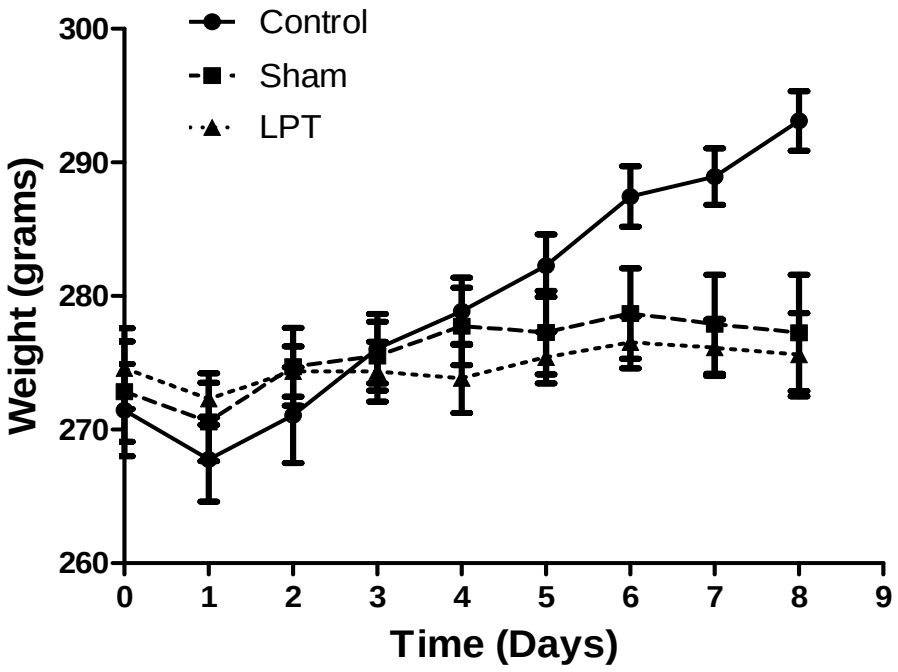

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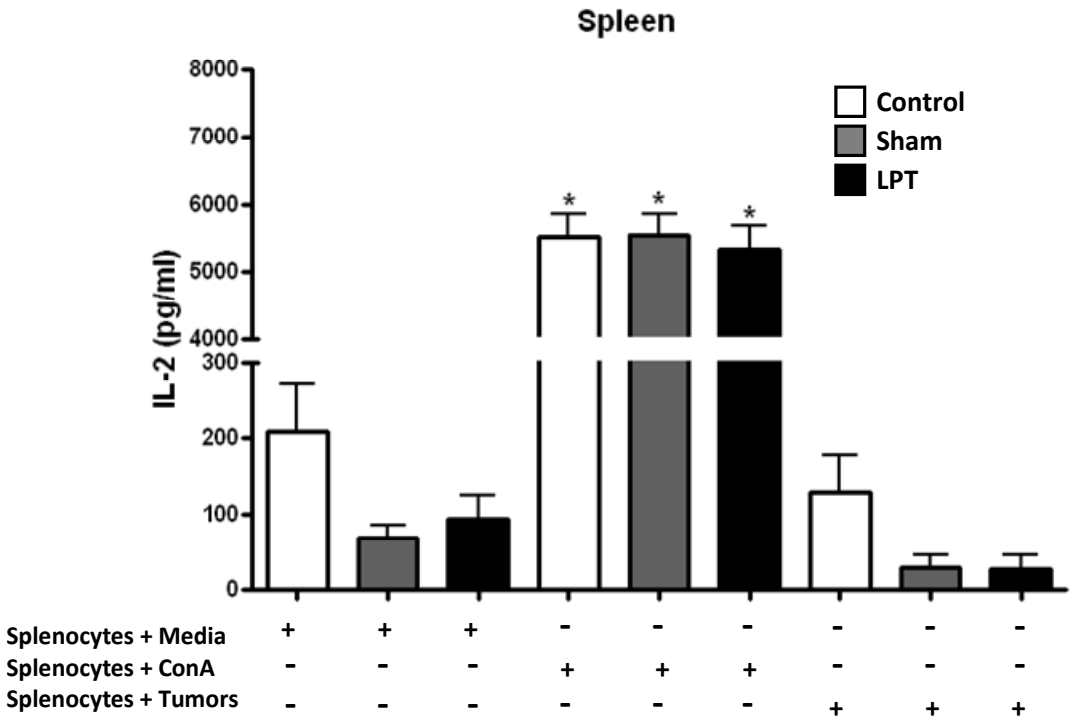

Figure 10. IL-2 cytokine is not produced by control, sham, or LPT splenocytes *in vitro*. On day 0, rats were intravenously injected with 1 x 10^6 MADB106 tumor cells through jugular vein catheters in 1ml sterile saline. On day 8 post-injection of tumor cells, splenocytes were collected and stimulated in a 96-well plate with ConA and MADB106 tumor cells for 48 hrs for subsequent IL-2 production assay. Data are means ± SEM of IL-2 pg/ml production (N=8 rats per group). * denotes $P < 0.05$

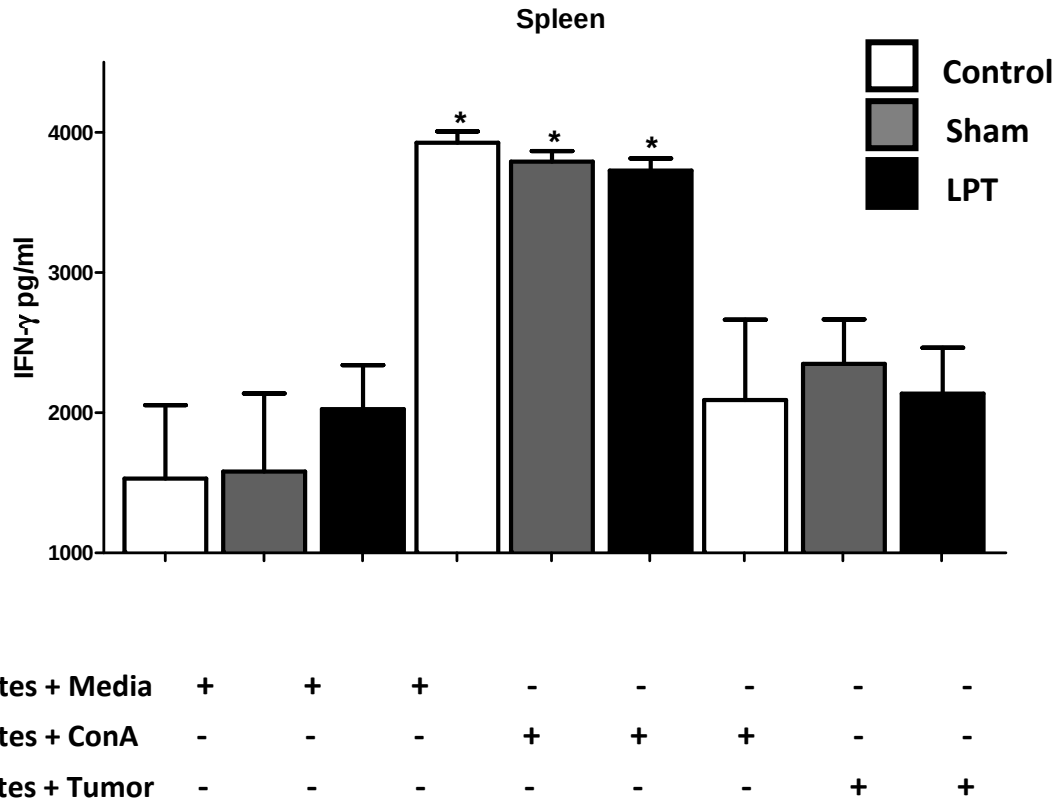

Figure 11. ***In vitro*** **IFN-γ production by splenocytes is not increased by LPT and is not induced by memory cells.** On day 0, rats were intravenously injected with 1 x 10^6 MADB106 tumor cells through jugular vein catheters in 1ml sterile saline. On day 8 post-injection of tumor cells, splenocytes were collected and stimulated in a 96-well plate with ConA and MADB106 tumor cells for 48 hrs for subsequent IFN-γ production assay. Data are means ± SEM of IFN-γ pg/ml production (N=4 rats per group). * denotes $P < 0.05$

CHAPTER I

INTRODUCTION AND BACKGROUND

Certain osteopathic manipulative treatments, such as high velocity low amplitude (HVLA) and lymphatic pump technique (LPT), are believed to be a contraindication in the presence of cancer with metastatic potential (1). The theory behind this belief is that by enhancing the movement of lymph through lymphatic vessels, LPT may accelerate the spread of cancer. However, unpublished data using a rat model indicate that LPT increases thoracic duct lymph flow, lymph leukocyte numbers, decreases bacteria numbers in the lungs, and enhances survival during pneumonia. Similarly, published studies have demonstrated that LPT enhances thoracic duct leukocyte flux and lymph leukocyte concentrations in the dog (2). Besides the direct effect of enhanced circulating leukocyte numbers produced by LPT, these cells may improve immune surveillance, potentially enhancing protection against infectious disease and cancer. In addition, leukocytes play an essential role in anti-tumor immunity, and interventions that augment the activity and number of leukocytes can reduce tumor mass (3, 4, 5). While the actual effects of LPT on cancer metastasis are still unknown, these data provide a strong rationale to test the efficiency of LPT at enhancing anti-tumor immune responses, which may in turn reduce tumor development and metastasis. Therefore, we hypothesize that administration of

LPT will prevent cachexia, reduce solid tumor formation, and enhance immunity in tumor-bearing rats.

The lymphatic system

The lymphatic system is responsible of collecting extracellular fluid from all parts of the body and returning it to the blood (6). Such extracellular fluid becomes lymph once it enters the lymphatic vessels, and it is continuously produced by filtration from the blood (6). As blood circulates under pressure, its fluid component, called plasma, diffuses out of the thin walls of capillaries into surrounding tissue (7). This fluid is called interstitial fluid and it permeates all tissues and bathes all cells (7). Lymphatic capillaries, the smallest lymphatic vessels, drain interstitial spaces and join to form a structure with branches that converge from all tissues (6, 7). The walls of lymphatic capillaries consist of a single layer of endothelial cells with large paracellular gaps between adjoining cells which easily allow the entry of interstitial fluid (6). In mammals, lymph vessels drain via a thoracic duct into the venous system (6). Therefore, fluid lost from the blood travels throught the lymphatic system and returns to the circulation (7). Lymph vessels contain unidirectional valves that only allow flow away from the lymphatic capillaries (6, 7). The larger lymphatic vessels are surrounded by smooth muscle which contracts rhythmically to create a low pressure that drives fluid away from the tissues (6). Lymph vessels are also compressed by contractions of the gut, skeletal muscles, and by general movements of the body, promoting lymph flow (6). In addition, lymph flows by intrinsic lymphatic contractility under autonomic control (7, 8).

Lymph flow is important in draining tissues of excess interstitial fluid. If the production of lymph exceeds lymph flow, edema can result. Moreover, the lymphatic system plays a critical

role in the body's defense against infection. When foreign antigen gains entry into the tissues, the lymphatic system transports the antigen to lymphoid tissues such as lymph nodes (7). As lymph is carried from tissues to lymphatic vessels, the fluid becomes progressively enriched with lymphocytes (7). Therefore, the lymphatic system transports both antigen and lymphocytes from tissues to organized lymphoid tissues where interaction between lymphocytes and trapped antigen occurs to initiate an immune response (7).

Breast cancer

Almost 1.5 million people in the United States are estimated to be diagnosed with cancer every year (9). Moreover, the incidence of numerous cancers, including breast cancer, is increasing (10). Cancer of the breast is a significant health problem from the time of diagnosis through the treatment and survivorship trajectory (11). In spite of improvements in the detection and treatment of breast cancer, approximately 40% of patients still die (10). The major cause of these deaths is the development of distant metastases which are generally detected at an incurable stage (10). Cancer treatments as well as the disease itself are a threat to the body, resulting in debilitating pathologic conditions.

One of the most distressing and debilitating complications of breast cancer treatment is lymphoedema (12). Lymphoedema is an abnormal accumulation of protein-rich excess fluid in the interstitial spaces (lymph stasis) (13). Despite current advances in treatment, many breast-cancer survivors still face permanent disruption of their lymphatic systems which in turn predisposes them to risk of secondary lymphoedema of the arm (13). Primary lymphoedema occurs by an inherent malfunction of the lymphatic vessels, whereas secondary lymphoedema occurs by an outside force such as tumors, effects of radiation, or removal of lymph nodes (14).

This chronic sequel of breast cancer has an impact on survivor's physical function, psychological distress and health-related quality of life (15). Therapies commonly used to treat secondary arm lymphoedema include physical therapy, manual lymphatic drainage, massage, pneumatic pumps, oral pharmaceuticals, laser therapy, compression bandaging and garments, limb exercises and limb elevation (16). However, there is little scientific supportive evidence for or against a particular physical treatment for lymphoedema (17).

Chronic lymph stasis can cause edema, accumulation of inflammatory mediators, and interference with immune cell trafficking. By disrupting trafficking of immunocompetent cells, lymph stasis impairs local immune surveillance (18). When the local mechanisms of immune surveillance become impaired, the affected region becomes an immunologically vulnerable area, predisposed for tumors and infections (14, 18). Hence, therapies which improve lymphatic drainage through the remaining functional lymph vessels are important to treat the effects of lymphoedema (14).

Lymphocyte circulation

Naive lymphocytes are mature B and T cells that have not yet encountered a specific antigen (6). These cells continually circulate from the blood into the peripheral lymphoid tissues and are then returned to the blood through the lymphatic vessels. The peripheral lymphoid organs include the lymph nodes, spleen, and the mucosal lymphoid tissues. The role of each of these tissues is to collect antigens ingested by dendritic cells or macrophages from the sites of infection and present it to recirculating lymphocytes, inducing an adaptive immune response (6).

Afferent lymphatic vessels carry antigen-bearing cells from sites of infection to the lymph nodes, in addition to draining fluid from tissues (6, 7). Lymph nodes are the site of B cell

activation and intense proliferation after encountering a specific antigen and the site of interaction between antigen-presenting cells and T cells, as well as the site of interaction between activated T cells and B cells upon specific antigen encounter (6). Once activated antigen-specific lymphocytes proliferate and differentiate, they leave the lymph nodes through the efferent lymphatic vessel to act as effector cells (6).

In 1959, Gowans et al. performed a series of pioneering studies on lymphocyte recirculation (19). Gowans developed a rat model for transfusing thoracic duct lymphocytes from a donor rat into the blood of a recipient rat. Thoracic duct lymph of the recipient rat was drained and intravenous transfusion of lymphocytes restored the thoracic duct output of lymphocytes to a normal level. Additionally, thoracic duct lymphocytes were radiolabeled prior to intravenous transfusion and the appearance of radioactivity in thoracic duct lymph occurred during the first 4 hours after transfusion. This study demonstrated that the output of lymphocytes from the thoracic duct is maintained by the continuous recirculation of lymphocytes from the blood back into the lymph.

Cancer and the immune system

Leukocyte trafficking is an essential mechanism of immune surveillance (20). The migration of immune cells to and from peripheral tissues enables them to survey the whole body, providing primary and secondary immune responses. Leukocyte recirculation through central and peripheral lymphoid organs and peripheral tissues is essential to maintain immune homeostasis (20). It is reasonable to investigate the efficiency of LPT at enhancing leukocyte trafficking and recruitment into cancer affected tissue with an attempt to elucidate modes of action as well as a new therapeutic approach.

The immune system has been shown to be an essential mechanism for the surveillance of oncogenesis and the elimination and control of established cancer (4). A network of both innate and adaptive immune cells is associated with anti-tumor immunity. Lymphocytes, including natural killer (NK) cells, natural killer T (NKT) cells, and T cells play an essential role in cancer immunosurveillance (5). NK cells are tumor suppressive innate immune cells that can recognize and eliminate tumor cells by several mechanisms (21). In addition to their cytotoxic function, NK cells regulate the adaptive immune response to cancer by activating dendritic cells and influencing T-cell differentiation (21). Ultimately, both NK cells and cytotoxic CD8+ T cells are responsible for the destruction of malignant cells (22). Another important subset of T lymphocytes found to be critical in tumor immunity is NKT cells. This unique subset of T-lymphocytes expresses NK cell markers as well as a T-cell receptor. The rapid response of NKT cells is characteristic of innate immunity and allows the polarizing cytokines to regulate adaptive immunity (23). NKT cells' perforin-mediated killing is also involved in the cytotoxicity against tumor cells (23). The networking and contribution of each of these populations is essential to anti-tumor immunity.

Osteopathic Manipulative Medicine

Osteopathic medicine is a healthcare profession similar to allopathic medicine but with a strong emphasis on the importance of the musculoskeletal system and normal body mechanisms as key factors for good health (1). Osteopathic philosophy maintains that the body has an intrinsic capacity to heal itself and that structure and function of the body is closely related (1, 24). Osteopathic physicians believe that one of the most effective ways to promote health is to remove obstructions to blood and lymph flow (24). A group of osteopathic manipulative therapies known as lymphatic pump techniques involve physical measures designed to enhance

lymph flow from distal body areas (1). Several lymphatic pump techniques have been developed since the foundation of osteopathy in 1874 by Andrew Taylor Still, MD (1). Among the lymphatic pump techniques used today include thoracic, abdominal, pelvic, splenic, liver, pancreatic, and pedal pumps (1, 24).

Increasing the rate of lymph flow through the use of lymphatic pumps techniques has long been thought to improve cellular activity and probably enhance immunity by mobilizing fluids, enhancing removal of metabolic waste, toxins, and bacteria (1). Several studies provide information on the mechanisms and importance of lymph circulation. Current research demonstrates that autonomically mediated, intrinsic lymphatic contractility plays a significant role in lymph propulsion, supporting the use of osteopathic manipulative techniques directed at influencing the autonomic nervous system to improve lymphatic circulation (25). An additional result of improved lymph flow is enhanced delivery of vaccine antigens, which improve the body's immunological defenses to combat infections (26, 27).

Since the early 1900's, osteopathic manipulative therapy (OMT) has been used as adjunctive therapy in the treatment of pneumonia (1, 28). During the 1918 Influenza-Pneumonia pandemic in the United States, the efficacy of OMT was strongly evaluated. The average mortality rate for patients with influenza complicated by pneumonia who were treated by allopathic physicians was approximately 25% (28). In contrast, the mortality rate for patients with influenza complicated by pneumonia who were treated with osteopathic manipulative therapy in addition to the prevailing therapy was 10% (28). This study provided evidence suggesting that OMT can treat infection and disease.

In the 1930's, Yale Castlio, DO, and Louise Ferris-Swift, DO performed one of the early studies that examined the effects of one of the lymphatic pump techniques on the immune system (29). One hundred healthy individuals were given splenic pump and blood was collected before treatment and at 5 and 30 minutes or at 10 and 60 minutes after splenic pump. Results from this study reported an increase in total leukocyte count in 81% of the subjects, an increase in opsonic index in 84% of the cases, and an increase in serum bacteriolytic power in 68% of the cases.

In 2005, Knott et al. demonstrated that both exercise and LPT produced significant net increases in thoracic duct lymph flow (30). Following that study, significant increases in leukocyte numbers in thoracic duct lymph by LPT was reported in a canine model (2). Data collected from these dog studies showed a 4-fold increase in lymph flow and a 2-fold increase in leukocyte numbers, causing an 8-fold net increase in leukocyte flux during LPT (2, 30). Together, these publications reveal that LPT increases thoracic duct lymph flow 4-fold and leukocyte numbers 2-fold, resulting in an 8-fold net increase in leukocyte flux during LPT. Similarly, pilot data demonstrate that moderate exercise increases thoracic duct leukocyte flux approximately 8-fold (unpublished data). Collectively, these data suggest that measures that enhance lymph can stimulate the mobilization of leukocytes into the lymphatic system, which may increase immune surveillance.

Several clinical studies provide evidence for the association of LPT with an increased number of blood leukocytes, antibody responses, immunization, shorter antibiotic therapy duration and hospital stays (29, 31, 32). Nevertheless, most of these clinical studies were pilot studies with varied types of LPT, age of subjects, antibodies analyzed and time points of sample collection.

From 1996-1998, Noll et al examined the effects of adjunctive osteopathic manipulative treatment (OMT) on elderly patients hospitalized with acute pneumonia (32). Subjects in the treatment group received a standardized OMT protocol treatment including thoracic lymphatic pump, while subjects in the control group received a standardized light touch sham treatment. Each treatment lasted 10-15 minutes and was given twice a day, 7 days a week until a study endpoint such as discharge from the hospital, ventilator-dependent respiratory failure, or death was reached. The study demonstrated that patients who received OMT treatment had a significantly shorter duration of intravenous antibiotic use, total antibiotic treatment, and length of hospital stay compared to control patients. In addition, on days 2 and 5, body temperature significantly increased in OMT treatment patients and significantly dropped in control patients. This data supports the idea that OMT may improve the immune response to infection as evidenced by higher fever (32).

Although reports on the clinical benefits of the lymphatic pump technique are numerous, little basic science research to validate the effectiveness of this osteopathic technique has been conducted.

Exercise and cancer

No studies have been reported that investigate the effects of LPT on anti-tumor immune responses; however, several studies suggest that moderate exercise promotes augmented immune function (3, 33, 34, 35). Specifically, exercise promotes an increase in the life span of tumor-bearing rats and a reduction in tumor mass (3). The numbers and activity of neutrophils, macrophages, and natural killer cells have been shown to increase with moderate exercise (33, 34, 35). Additionally, exercise can increase lymphocyte activation and proliferation during

cancer (3). These studies suggest that exercise increases anti-tumor immune responses, which reduce tumor load and increase survival. Therefore, like exercise, LPT may enhance leukocyte release into the lymphatic system, enhancing immune surveillance and anti-tumor immune responses.

Model to study the effects of LPT on cancer

To measure the effects of LPT on various diseases, a rat model was developed in our laboratory to specifically assess the effects of LPT on the lymphatic and immune system. To determine if application of LPT to the rat would increase thoracic duct lymph flow and leukocyte numbers similar to measurements in the dog, rats were anesthetized and a catheter was inserted into the thoracic duct. Application of LPT to the rat resulted in a 2-fold increase of total leukocyte numbers and a 3-fold increase in lymph flow. These results were consistent to studies using the dog model (2, 30), and demonstrate that application of LPT in both animal models has a similar effect on lymphatic flow and leukocyte release.

To determine if LPT has an immunological effect on breast cancer, a MADB106 tumor cell model was utilized. MADB106 is a chemically induced adenocarcinoma originally isolated from the lung of a Fisher 344 rat. Moreover, it is a natural killer cell (NK) sensitive mammary adenocarcinoma that metastasizes to the lung, and is commonly used to study the effects of tumor metastasis in rats (36, 37, 38). This tumor model has been used in several studies to investigate the effects of NK cells on tumor metastasis in syngeneic models (37). Intravenous administration of MADB106 tumor cells results in lung tumor retention by approximately 3 days post-injection, whereas subcutaneous implantation induces the development of primary tumors within 10 days (36, 39, 40, 41). Previous studies demonstrate the significant role of NK cells in

controlling MADB106 lung tumor retention (40). However, there is additional evidence supporting the antitumor activity of B lymphocytes in the lungs of this rat model (38, 39). Administration of antibody against NK cells for the depletion of these cells results in decreased lung tumor clearance shortly after tumor inoculation (38, 40). Similarly, immunoneutraliztion of B lymphocytes generate an increase in lung metastases in this experimental model (38, 39). In addition, removal of lung B cells from total lung lymphocyte cultures reduce IFN-γ production in the presence of MADB106, suggesting the important antitumor influence of B lymphocytes given the proactive anti-tumor role of IFN-γ (38).

Significance

In conclusion, results from preliminary studies suggest that both LPT and exercise enhance the lymphatic release of leukocytes. The increased number of circulating leukocytes produced by LPT may improve immune surveillance. Therefore, it is reasonable to investigate the effectiveness of LPT at increasing anti-tumor immunity and provide protection against tumor development and metastasis. It is critical to investigate and provide scientific evidence for the effect of LPT in patients with cancer in order to support this osteopathic treatment as a valuable cancer therapy. Also, if these studies demonstrate that LPT has no effect on tumor development and metastasis, then the lymphatic pump technique could be used to treat lymphoedema which is a complication of breast cancer.

CHAPTER II

PILOT EXPERIMENTS TO DEVELOP A RAT MODEL FOR THE STUDY OF LPT EFFECTS ON CANCER

Introduction

While most studies on the lymphatic pump treatment (LPT) have been performed with humans with infectious and respiratory diseases, a few animal models have also been used to analyze the effects of this osteopathic treatment (2, 32, 42). In 2000, Dery et al. examined the effects of LPT on lymph flow in rats (42), while Knot et al. 2005 measured the effects of LPT on thoracic duct lymph flow in dogs (32). Furthermore, in 2007, Hodge et al. examined leukocyte count and flux in the thoracic duct lymph of dogs (2). However, the effect of LPT on tumor development and metastasis has never been tested. Therefore, in order to initiate these studies, it was necessary to develop a small animal model.

Originally, a rat animal model was developed to examine the effects of LPT application on the lymphatic and immune system. First, to determine if LPT on rats would enhance thoracic duct lymph flow and leukocyte numbers as previously demonstrated in a dog model (2, 32), a pilot study was conducted. Rats were anesthetized and a catheter was placed in the thoracic duct while LPT was applied. Results from that study confirmed that LPT has a similar effect on

lymphatic flow and leukocyte release in the rat. Application of LPT to both the dog and rat can increase thoracic duct lymph flow and leukocyte concentrations in lymph approximately 4-fold. Therefore, this current study aimed to further develop the rat model for the analysis of LPT effect on cancer using a tumor cell line that metastasizes to the lung.

Material and Methods

Rats. Male inbreed Fisher 344 rats, weighing 250-350 grams were used in the preliminary studies (Charles Rivers). Rats were housed and fed ad lib accordingly to the Institutional Animal Care and Utilization Committee (IACUC) of the University of North Texas Health Science Center in the barrier facility.

Tumor cell culture. The tumor cell line used in these studies was MADB106, which is a chemically induced adenocarcinoma originally isolated from the lung of a Fisher 344 rat (36, 37, 38). MADB106 cell line was maintained in monolayer cell culture with 5% CO_2, at 37°C, in RPMI complete medium supplemented with 10% heat inactivated fetal bovine serum, 1X antibiotic/antimycotic, 1X L-glutamine, and 1X HEPES.

Tumor inoculation. Under isoflurane gas anesthesia, 10 x 10^6 CFSE-labeled and unlabeled MADB106 mammary adenocarcinoma cells were intravenously injected through the tail vain. Tumor cells were injected in a volume of 1mL sterile PBS. MADB106 cells were maintained and injected in log phase.

Application of control, sham treatment and lymphatic pump treatment. Twenty four hours after MADB106 intravenous inoculation, rats were divided into control, sham, or LPT treatment groups. Control animals received no treatment or anesthesia, sham animals received 4 minutes of light touch under 10mg/kg of propofol anesthesia and LPT animals received 4 minutes of

lymphatic pump under 10mg/kg of propofol. Each treatment was given for 6 consecutive days. For the application of LPT, a rhythmic bilateral compression of the abdomen below the costal angle in a craniodorsal direction was applied until significant pressure was felt against the diaphragm of rats. No effort was made to exceed the anatomic range of motion. The compressions were applied daily under propofol anesthesia for 4 minutes at a rate of 1 Hz for 6 days.

Lymphocyte isolation. To collect lung single-cell suspensions, lung tissue was placed in RPMI wash media (5% FBS, 1X antibiotics/antimycotics, 10mM Hepes) and finely minced for subsequent tissue dissociation in a gentleMACS (MACS Miltenyi Biotec). Next, lung cell suspensions were passed through a nylon mesh filter to remove non-dissociated tissue. After lung cell suspensions were washed twice with RPMI wash media, they were gently layered over Lympholyte Rat gradient (Cedarlane Laboratories Limited) to purify lymphocytes by density gradient centrifugation. Samples were centrifuged at 1200g for 20 minutes, 25°C, slow break. The lymphocyte layer separated by the gradient was collected for cell counting by hemacytometer.

Leukocyte counts. To prepare lung tissue samples for leukocyte enumeration, tissue samples were treated as described in the previous section. A hemacytometer (Bright-Line) and microscope was used to count total lung leukocytes. Lung samples were vortexed and 2µL of lung was added to 18µL of PBS (Hyclone). The solution of lung and PBS was gently mixed with 20µL of Trypan Blue (Sigma). Next, 10µL of the solution was placed on the hemacytometer slide and the middle square was used to calculate the total number of leukocytes.

MADB106 cell count. Lung samples were analyzed for the presence and number of MADB106 mammary adenocarcinoma cells. Lung samples were prepared as previously described, except MADB106 viable cells were counted on a hemacytometer (Bright-Line) prior to Lympholyte Rat gradient centrifugation. Lung samples were vortexed before the addition of 2µL of sample into 18µL of PBS (Hyclone). The solution of lung and PBS was gently mixed with 20µL of Trypan Blue (Sigma). Next, 10µL of the solution was placed on the hemacytometer slide and the middle square was used to calculate the total tumor cells.

CFSE-tumor labeling. Cells were harvested from culture flasks by using sterile PBS and a cell scraper to remove cells from the surface of flasks. Tumor cells were washed twice and suspended in sterile PBS for hemacytometer cell count. Tumor cell viability was determined by Trypan blue staining. Subsequent to cell count, cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye using a commercially available kit (Sigma-Alderich) prior to intravenous injection in order to investigate tumor localization. Briefly, tumor cells were incubated with CFSE for 10 minutes at 37°C. Ice cold culture media was added and cells were incubated for an additional 5 minutes on ice. Cells were washed twice in sterile PBS and rats were inoculated. Following 5 days after cancer induction, lungs were removed and microscopic slides of lung tissue homogenates were prepared for fluorescence microscopy.

Results

Intravenous injection of MADB106 causes tumor cell retention and formation of solid tumors in pulmonary tissue by day 5. To monitor the establishment of tumor cells in pulmonary tissue after MADB106 inoculation via the tail vein, 10 x 10^6 MADB106 tumor cells were labeled with the vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE). Five and

seven days after intravenous tumor inoculation, the presence of CFSE-labeled tumor cells in the lungs was confirmed by fluorescence microscopy (Figure 1). This result demonstrates that MADB106 mammary adenocarcinoma cells localize to the lungs after intravenous injection.

Intravenous injection with MADB106 increases pulmonary leukocyte numbers. To investigate the effect of intravenous tumor cell injection on lung leukocyte numbers, lungs removed at 5 and 7 days post-injection were compared to naive lungs which served as negative controls (Day 0). Total lung leukocyte numbers significantly increased after intravenous injection of MADB106 tumor cells (Figure 2). This data indicates that dissemination of MADB106 tumor cells to the lung significantly increase leukocyte numbers in lung tissue.

Propofol anesthesia does not alter the pulmonary leukocyte environment. To test whether propofol would alter the pulmonary immune cell environment, healthy rats received control, sham or LPT treatment for 7 days. Sham and LPT were applied under propofol anesthesia. Repeated injection of propofol anesthesia did not increase leukocyte entry into pulmonary tissue (Figure 3). Furthermore, there were no differences in leukocyte numbers between control, sham and LPT, suggesting that an inflammatory signal is necessary to promote retention of leukocytes in the lung. This data shows that propofol is a suitable anesthesia method to use in our model.

Discussion

Results from preliminary studies demonstrate that intravenous injection of MADB106 mammary adenocarcinoma results in distribution of tumor cells into the lungs as well as the establishment of solid tumors in lung tissue by day 5. In previous rat studies conducted in our laboratory, isoflurane gas anesthesia had been used for the application of sham and LPT treatment. However, data from those experiments demonstrate that the administration of isoflurane gas anesthesia during either sham or LPT treatment increases leukocyte trafficking

into the lungs of both tumor bearing and healthy rats. This result suggests that isoflurane anesthesia has the intrinsic capacity to increase the number of leukocytes in the lungs in the presence and absence of lung disease. The specific mechanisim responsible for the gas anesthesia-induced effects on the pulmonary environment of the rat is uncertain.

Propofol has been reported to have less immunological effects compared to gas anesthesia (36). Since it is necessary to use anesthesia to apply LPT to the rat, propofol anesthesia was used in this cancer model. The repeated use of propofol during sham and LPT does not induce leukocyte trafficking into the lungs of healthy animals. Thus, propofol was identified as an appropriate anesthetic for the application of sham and LPT treatment. Moreover, since LPT is performed under anesthesia for 7 consecutive days, rats with indwelling jugular vein catheters were used in subsequent studies. By using cannulated rats, the need to repeatedly inject rats with anesthesia was eliminated thus minimizing pain and distress.

In conclusion, results from preliminary studies demonstrate that intravenous injection of MADB106 results in tumor cell dissemination to the lung by day 5, and the presence of tumors in pulmonary tissue remarkably increase leukocyte trafficking into the lungs. Moreover, it was identified that propofol is a suitable anesthesia to use during the application of sham and LPT treatment.

CHAPTER III

LYMPHATIC PUMP TREATMENT INCREASES PULMONARY IMMUNITY AND DECREASES SOLID TUMOR FORMATION IN THE LUNG

Introduction

The lymphatic pump treatment (LPT) is an osteopathic manipulation designed to enhance lymph flow and promote health (1, 24). LPT has been historically used as adjunctive therapy in patients with infectious and respiratory diseases since the early 1900s (1, 24, 28). Some of the reported clinical benefits include an increase number of blood leukocytes, increased antibody responses, enhanced immunization, as well as shorter antibiotic therapy duration and shorter hospital stays. Lymphatic pump techniques have long been thought to improve cellular activity by mobilizing fluids, enhancing removal of metabolic waste, and possibly boosting immunity (24). However, less emphasis has been placed on more basic research in this osteopathic manipulation.

In our preliminary cancer studies, the differences between sham and LPT treatment were unclear due to the evident effect of isoflurane gas anesthesia on the lung environment of these animal groups. Consequently, a pilot study was conducted to investigate the effect of another anesthetic which has been reported to induce less immunologic effects compared to gas

anesthesia. Specifically, the effect of intravenous administration of propofol anesthesia on the pulmonary immune cell environment and the effect of LPT application under this anesthetic were determined. Once it was confirmed that propofol did not cause a change in the lung cell environment, the following experiments were conducted to focus on one specific aim. The specific aim was to determine if LPT prevents or promotes lung tumor development and if it enhances immune cell activity within the lungs of rats with cancer.

Material and Methods

Rats. Immune competent male inbred Fisher 344 rats with indwelling jugular vein catheters weighing 250-350 grams were used in these studies (Taconic). Catheterized rats were used in order to avoid repeated administration of intraperitoneal anesthesia, minimizing pain and distress. Rats were housed and fed accordingly to the Institutional Animal Care and Utilization Committee (IACUC) of the University of North Texas Health Science Center in the barrier facility and the study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*. A minimum of five rats were used per group of control, sham, and lymphatic pump treatment and four separate experiments were performed. Rat weights from control, sham, and LPT treatment groups were recorded from day 0-8.

Tumor cell culture and inoculation. The MADB106 mammary adenocarcinoma cell line was maintained in monolayer cell culture with 5% CO_2, at 37°C, in complete culture media (RPMI 1640) supplemented with 10% heat inactivated fetal bovine serum, 1X antibiotic/antimycotic, 1X L-glutamine, and 1X hepes. To prevent possible mutation of the cell lines, cell cultures were limited to 5 passages (from the original batch) before use in the experiments. Cells were harvested from culture flasks by using sterile PBS and a cell scraper to remove cells from the surface of flasks. Tumor cells were washed twice and suspended in sterile PBS for

hemacytometer cell count prior to being injected into animals. Tumor cell viability was determined by Trypan blue staining. Rats were intravenously injected with 1 x 10^6 MADB106 mammary adenocarcinoma cells in log phase in a volume of 1ml sterile PBS through the jugular vein catheter.

Application of control, sham and lymphatic pump treatment. Rats were divided into control, sham, or LPT treatment groups 24 hours following intravenous injection of tumor cells. For the application of sham and LPT treatment, rats received 10mg/kg of propofol anesthesia via the jugular vein catheter. Under anesthesia, each rat in the LPT treatment group received 4 minutes of lymphatic pump daily for 7 days. To perform LPT, a rhythmic bilateral compression of the abdomen below the costal angle in a craniodorsal direction was applied until significant pressure was felt against the diaphragm of rats. No effort was made to exceed the anatomic range of motion. The compressions were applied for 4 minutes at a rate of 1 Hz. Sham rats were anesthetized and lightly touched for the same amount of time than the LPT rats to serve as the anesthetic control. In contrast, control rats were intravenously infused with tumor cells but were not anesthetized nor given sham or lymphatic pump treatment. The overall health and weights of rats in each group was recorded from day 0-8.

Lung tumor metastases assessment. After 7 days of control, sham, or LPT treatment, rats were killed by the opening of the chest cavity under ketamine and xylazine anesthesia. To quantify tumor metastases, a method described by Wexler (43) was used. Lungs were removed and infused with a distilled water solution containing 15% India ink via the trachea. Next, lung tissue was placed in dedying solution (70% ETOH, 10% formaldehyde, 5% gliacial acetic acid, 15% distilled water) for 48 hours. Normal lung tissue stains black, while tumors are exposed as white nodules on the surface of lungs. Metastatic tumor nodules, which resist the dye, were

macroscopically enumerated on lung surface. For the counting procedure, individual lung lobes were separated and the number of white nodules in each lobe was recorded. For each animal, the number of tumor nodules in individual lobes was added to quantify solid tumors on the surface of the entire lung.

Lymphocyte isolation. Eight days following intravenous tumor cell injection, the lungs and spleens and were removed. For the preparation of lung single-cell suspensions, lung tissue was placed in RPMI wash media (5% FBS, 1X antibiotics/antimycotics, 10mM HEPES) and finely minced for subsequent tissue dissociation in a gentleMACS (MACS Miltenyi Biotec). Next, lung cell suspensions were passed through a nylon mesh filter to remove non-dissociated tissue. After lung cell suspensions were washed twice with RPMI wash media, they were gently layered over a Lympholyte Rat gradient (Cedarlane Laboratories Limited) to purify lymphocytes by density gradient centrifugation. Samples were centrifuged at 1200 g for 20 minutes, 25°C, slow break. The lymphocytes separated on the gradient were collected for enumeration using a hemacytometer. In addition, flow cytometry analysis of lymphocyte subsets was performed.

Single-cell suspensions of spleen cells were prepared by mashing spleens through a nylon mesh filter. In order to remove red blood cells, ACK lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA) was used. Spleen cells were collected and washed twice in RPMI wash media (5% FBS, 1X antibiotics/antimycotics, 10mM Hepes). The spleen lymphocytes were used for Hemavet total cell count, flow cytometry analysis of lymphocyte subsets, IFN-γ and IL-2 enzyme-linked immunosorbent assays (ELISA).

Leukocyte counts. Blood, lung and spleen samples were analyzed for leukocyte numbers. To prepare lung and spleen tissue samples for enumeration, tissue samples were treated as described

in the previous section. Blood samples were collected via the jugular vein catheters on the day of tumor cell inoculation and after 7 days of treatment (approximately 0.5ml). Total leukocytes and differential leukocyte counts in each blood and spleen sample were determined using a Hemavet 950 Cell Analyzer (Drew Scientific). A hemacytometer (Bright-Line) and microscope was used to enumerate lung total leukocytes. For hemacytometer count of lung samples, the lung sample was vortexed and 2µL of lung was added to 18µL of PBS (Hyclone). The solution of lung and PBS was gently mixed with 20µL of Trypan Blue (Sigma). Next, 10µL of the solution was placed on the hemacytometer slide and the middle square was used to calculate the leukocytes.

MADB106 cell counts. Lung, spleen and liver samples were analyzed for the presence and number of MADB106 mammary adenocarcinoma. Lung samples were prepared as previously described, except MADB106 viable cells were counted on a hemacytometer (Bright-Line) prior to Lympholyte Rat gradient centrifugation. The lung sample was vortexed and 2µL of lung was added to 18µL of PBS (Hyclone). The solution of lung and PBS was gently mixed with 20µL of Trypan Blue (Sigma). Next, 10µL of the solution was placed on the hemacytometer slide and the middle square was used to calculate the total tumor cells.

Similarly, viable MADB106 cells were enumerated in spleen samples using a hemacytometer. After spleen homogenates were centrifuged and washed once with RPMI wash media, samples were prepared for tumor cell count. For spleens, 5µL of spleen sample was added to 20µL of PBS. The spleen and PBS solution was then gently mixed with 25µL of Trypan Blue. 10µL of the solution was placed on the hemacytometer slide and the middle square was used to determine the total tumor cells.

Liver tissue was dissected out and small pieces of each lobe were removed and placed in RPMI wash media. Single-cell suspensions of liver cells were prepared by mashing liver tissue through a nylon mesh filter. Next, 5µL of liver sample was added to 20µL of PBS and the solution was mixed with 25µL of Trypan Blue. MADB106 cells in the liver were enumerated in the hemacytometer as described before.

Cell Culture conditions. Spleen lymphocytes were cultured in 96-well flat-bottomed microtiter plates in RPMI 1640 (HyClone) supplemented with 10%FBS (HyClone), antibiotic/antimycotic solution (Sigma), L-glutamine (Cellgro), and Hepes (Fisher Scientific). Lymphocytes were co-cultured at a concentration of 1 x 10^6 cells/well, in the presence or absence of 1 x 10^4 MADB106 cells/well, or 10 mg/mL of concanavalin A in a volume of 200 µL/well of culture media. Lymphocytes were incubated for 48 hours at 37°C and 5% CO_2. Stimulation supernatants were collected and stored at -80°C until analyzed for cytokine levels.

Cytokine ELISA assay. The amount of cytokine production in spleen culture supernatants and cardiac blood serum was determined by capture ELISA (enzyme-linked immunosorbent assay). Rat interferon-gamma (IFN-γ) cytokine was measured using rat IFN-γ ELISA kits (BD Biosciences). In summary, flat-bottom 96-well microtiter plates were coated overnight at room temperature with 100µL of capture antibody. Plates were then washed 5 times using wash buffer (PBS with 0.05% Tween-20) and blocked for 1 hour with 200µL of assay diluent (PBS with 10% FBS) to prevent non-specific binding. After blocking the plates, 100µL of spleen culture supernatant, blood serum or IFN-γ standard cytokine was placed into appropriate wells. Following overnight incubation at 4°C, plates were washed 5 times using wash buffer. 100µL of enzyme reagent (Streptavidin-horseradish peroxidase conjugate (SAv-HRP)) was added to each well and incubated for 30 minutes at room temperature. Plates were washed with wash buffer 7

times before 100µL of substrate solution (BD Pharmingen TMB Substrate Reagent Set) was added to incubate plates for approximately 30 minutes at room temperature in the dark in order to detect the cytokine content. Subsequently, 50µL of stop solution (0.25M HCl) was added to each well and plates were read at an absorbance of 450nm using a MRX microplate reader (Dynatech Laboratories). Cytokine contents were determined by comparing standard curves from IFN-γ protein standard using quadratic linear regression analysis.

Interleukin-2 (IL-2) cytokine contents were measured using rat IL-2 ELISA kits (R&D Systems). Briefly, flat-bottom 96-well microtiter plates were coated overnight at room temperature with 100µL of capture antibody. Plates were washed using wash buffer (PBS with 0.05% Tween-20) for a total of 3 times and blocked with 300µL of reagent diluent (R&D Systems) for 1 hour at room temperature. Wash was repeated and 100µL of spleen culture supernatant or IL-2 standard cytokine was added into appropriate wells for an incubation of 2 hours at room temperature. Plates were washed one more time for a total of 3 times with wash buffer to remove any unbound antigen. 100µL of detection antibody was then added and incubated for 2 hours at room temperature. Wells were washed as mentioned previously and 100µL of Streptavidin-HRP (R&D Systems) was added to each well for 20 minute incubation in the dark. Following another wash, 100µL of substrate solution (R&D Systems) was added to each well and incubated in the dark for 20 minutes. Finally, 50µL of stop solution (0.25M HCl) was added and plates were read at an absorbance of 450nm using a MRX microplate reader (Dynatech Laboratories). Cytokine content was determined by comparing standard curves from IL-2 protein standard using quadratic linear regression analysis.

Flow cytometry. Immunofluorescent staining of lung and spleen lymphocytes was performed using monoclonal antibodies against CD3+ T cells, CD4+ T cells, CD8+ T cells, CD49d, CD61,

macrophages, granulocytes, B cells, and NK cells. For each stain, 1 x 10^6/100µL of lung or spleen cells were incubated with the optimal concentration of monoclonal antibody for 30 minutes in the dark at room temperature. Individual lung and spleen cell aliquots were stained with either 20µL of anti-rat CD3-FITC isotype control, or 5 µL of anti-rat CD4-RPE isotype control. Additionally, separate 100µL aliquots of each sample were stained with 2µL of CD3, 2.5µL of CD4, 2.5µL of CD8, and 10µL of CD49d or CD61. Separate aliquots were stained with 2µL CD3 and 2.5µL of CD4, 2µL of CD3 and 2.5µL of CD8, 10µL of CD49d and CD61, 20µL of MO, 2µL of B cell, and 2µL of NK cell. All flow cytometry antibodies were obtained from BioLegend, except CD49d and CD61 from SeroTec. One 100µL lung and spleen aliquot was left unstained to serve as a negative. Individual 100µL aliquots of each of the samples were washed two times with 2mL of staining buffer (PBS with 2% fetal bovine serum) at 400g, 5 minutes, 4°C. Following removal of supernatant, lung and spleen cells were fixed with 0.5% paraformaldehyde (Sigma) in PBS and stored at 4°C until flow cytometry analysis.

Cells were then analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter). Lymphocyte gates and detector voltages were set using unstained lung and spleen cell controls. Cell populations were detected from histogram peaks or dot plot cell clusters. Each cell population was expressed as the percentage of the number of stained cells. To calculate the total number of a specific lymphocyte population, their percentage was multiplied by the total number of cells in lung or spleen samples as determined by hemacytometer or hemavet count.

Statistical analysis. Results obtained from control, sham and LPT groups were compared. To perform statistical analysis, results were analyzed by ANOVA to compare sham and control treatment values with LPT values. GraphPad Prism version 5.00 for Windows, GraphPad

Software was used. Differences among mean values with $P \leq 0.05$ were considered statistically significant. Data was expressed as mean ± SEM.

Results

LPT reduces pulmonary solid tumors. To determine if LPT would reduce the number of solid tumors in the lungs, rats were intravenously injected with tumor cells and given control, sham, or LPT treatment from day 1-7. Application of LPT treatment significantly reduced solid tumors in the lungs compared to control and sham treatment (Figure 4). This finding suggests that LPT is either protecting against the establishment of lung tumors or may promote the dissemination of tumors to other organs. Therefore to rule out the possibility that reduction of lung tumors in LPT rats was due to extra-pulmonary MADB106 dissemination, the spleen and liver were examined for the presence of tumor cells after 7 days of control, sham, or LPT treatment application. Intravenous injection of MADB106 mammary adenocarcinoma resulted in tumor cells in the spleen. However, the presence of tumor cells in spleens was not surprising since this organ serves as a filter element for cells, proteins, etc that enter the blood circulation (6). Importantly, the total number of tumor cells in the spleen of LPT animals was not significantly different than control and sham (Figure 5). Furthermore, no tumor cells were found in the liver of any treatment group. The results from these experiments indicate that LPT is able to reduce the development of solid tumors in the lung, without inducing tumor dissemination.

LPT increases leukocyte numbers in tumor bearing lungs. To determine if the reduction of solid tumors in the lungs of animals given LPT was associated with an increase in the number of leukocytes in the lungs, rats were intravenously injected with tumor cells and control, sham, or LPT treatment was applied as previously described. On day 8, lungs were removed and total

leukocytes, macrophages, NK cells, B cells, $CD4^+$ T cells and $CD8^+$ T cells were measured. Lymphatic pump treatment significantly increased pulmonary leukocytes approximately 2-fold compared to control and sham (Figure 6). As illustrated in Table 1, a 2-fold significant increase in lung macrophages was induced by LPT in contrast to both control and sham treatment. Furthermore, LPT application resulted in a 3-fold increase of NK and B cells and an approximate 2-fold increase in $CD4^+$ T cells and $CD8^+$ T cells. Leukocytes play an essential role in the surveillance of oncogenesis and the elimination and control of established cancer (4, 5). These results indicate that LPT may have enhanced pulmonary trafficking of leukocytes with anti-tumor activities, thereby inhibiting tumor growth in the lungs.

LPT increases trafficking of gastrointestinal derived lymphocytes into tumor bearing lungs. Our laboratory has previously demonstrated that LPT mobilizes leukocytes from gut associated lymphoid tissue (GALT) into lymphatic circulation (44). Antigen specific lymphocytes primed in gastrointestinal tissue can migrate to the respiratory tract and provide protection during infection or inflammation (45). Therefore, in order to investigate if LPT enhanced the trafficking of GALT derived lymphocytes into the lungs with tumors, lung leukocytes were stained for the gastrointestinal homing receptor integrin α4β7. The α4β7 integrin is an adhesion molecule involved in several types of cell-cell interactions, such as cell recruitment and lymphocyte activation (46). Lymphocytes and monocytes express this adhesion molecule and expression is upregulated upon cell activation. Studies demonstrate the role of α4β7 integrin in the migration of lymphocytes into mucosal tissues (46). LPT increased the numbers of gastrointestinal lymphocytes in pulmonary tissue by approximately 1.5 fold (Figure 7) compared to control and sham treatment. This result is consistent with ongoing studies in our

laboratory utilizing a dog model, suggesting that the GALT is a tissue source of the lymphocytes released during LPT treatment.

LPT increases serum IFN-γ cytokine. IFN-γ production plays a critical role in the development of powerful anti-tumor effector functions mediated by both adaptive and innate immunity through its capacity to promote the generation of tumor-specific $CD4^+$ Th1 T cells and cytolytic T cells (CTL), plus activate cytolytic activity in macrophages and NK cells (5, 38). To examine the concentration of IFN-γ in serum, rats were euthanized and cardiac blood was collected 8 days after MADB106 intravenous injection. Serum from the blood of LPT rats contained a higher concentration of IFN-γ than control and sham rats (Figure 8). Data from this experiment indicates that application of lymphatic pump may be inducing a moderate increase in the peripheral response against MADB106 tumor cells.

Sham and LPT treatment inhibit weight gain. One day post tumor inoculation, rats in each treatment group lost weight (Figure 9); however by day 2 post-injection, rat weights returned to day 0 values. This finding indicates that MADB106 induces cachexia during the first few days of tumor cell establishment into pulmonary tissue. Even though rats in each treatment group gradually gained weight from day 2-8, application of either sham or lymphatic pump treatment (LPT) during 7 consecutive days prevented considerable weight gain in those animal groups in contrast to control animals (Figure 9). It is possible that the daily administration of propofol anesthesia to sham and LPT rats caused nausea, or rats in those two treatment groups may be experiencing higher levels of handling stress, thus decreasing weight gain.

LPT does not increase IL-2 and IFN-γ cytokine production *in vitro*. To investigate the tumor-specific *in vitro* cytokine production of spleen leukocytes, rats were euthanized and splenocytes

co-cultured for 48 hours with ConA or MADB106 tumor cells. *In vitro* stimulation of spleens isolated from control, sham, and LPT rats produced no significant concentration of interleukin-2 (IL-2) cytokine when incubated with or without tumor cells (Figure 10). Low IL-2 production in control, sham or LPT treatment group suggests that the tumor response observed 8 days post tumor injection is most likely mediated by innate immune cells and that LPT does not induce cell proliferation. In addition, the lower concentration of IL-2 cytokine in sham and LPT rats compared to control suggests that propofol or stress may be inhibiting proliferation of T lymphocytes. Similarly, interferon-gamma (IFN-γ) cytokine production by spleen leukocytes in response to ConA and MADB106 incubation for 48 hours was analyzed in control, sham, and LPT rats. *In vitro* stimulation of spleens isolated from LPT rats produced similar concentrations of IFN-γ in response to culture with MADB106 as control and sham rats (Figure 11). Moreover, production of IFN-γ by MADB106-stimulated splenocytes of LPT rats was similar to the cytokine production of splenocytes incubated in culture media alone (Figure 11). Data collected from this experiment suggests that application of lymphatic pump does not enhance splenocyte IFN-γ production *in vitro.* Furthermore, while MADB106 mammary adenocarcinoma induces IFN-γ production by splenocytes *in vitro*, this tumor response is not induced by antigen-specific memory cells.

Discussion

Historically, the use of lymphatic techniques is considered a contraindication in the presence or potential presence of metastatic cancer (1). Although many osteopathic physicians use manipulation as an adjunctive therapy to treat many illnesses, no large controlled trials for the effectiveness of manipulation for conditions other than lower back pain exist (1). The potential of LPT to induce tumor dissemination was investigated in this study. Importantly, our

animal model provided scientific evidence that application of LPT during cancer does not promote tumor dissemination.

This study shows that administration of lymphatic pump treatment increases leukocyte entry into the lungs by approximately 2 fold over control and sham. It is thus possible that the increase of total lung leukocytes induced by LPT could be, at least in part, responsible for the significant reduction of solid tumors observed in pulmonary tissue of LPT animals. By increasing leukocyte trafficking into affected tissue, LPT may facilitate leukocyte and antigen interaction, hence enhancing the development of an immune response. Furthermore, the observed redistribution of GALT derived leukocytes into the lungs upon application of LPT may further enhance protection against tumor establishment.

Recognition of tumor antigens by the immune system triggers an immune response to eliminate malignant cells. NK cells have the capacity to lyse NK-sensitive tumor cells by releasing perforin and granzyme-rich granules without prior encounter with tumor targets. Since NK cells are not MHC restricted, they can eliminate tumor cells that do not express the MHC complex and induce apoptosis in a variety of malignant cells (47). Application of LPT resulted in a significant increase of NK cells in tumor bearing lungs. Although LPT treatment increased NK cells in the lungs, the low number of cells observed at 8 days after tumor inoculation suggests that NK cells may have performed their innate immunity effector functions against MADB106 days earlier during the time course of the study. Higher number of NK cells may be present in the lungs a few days after intravenous tumor injection.

Macrophages present in the tumor microenvironment phagocytose apoptotic tumor-cell remnants and may process these proteins for subsequent presentation to T cells (47). The

remarkable increase of macrophages in the lungs of LPT rats may represent the activation of these cells by previous NK cell production of IFN-γ cytokine. Macrophages appear to preferentially stimulate Th1 cell activation (46). Therefore, enhanced number of activated macrophages by LPT may be processing tumor antigens to induce a stronger adaptive immune response.

Helper T cells ($CD4^+$) only respond to antigen presented on class II MHC proteins expressed by antigen-presenting cells, respectively, dendritic cells, macrophages, and B-cells (7, 47). Following activation, $CD4^+$ T cells release IL-2 that binds to IL-2 receptors on cell surface of NK cells and $CD4^+$ T cells. IL-2 cytokine augments the cytotoxic activity of NK cells while it induces proliferation of activated $CD4^+$ T cells. Effector cells interact with tumor cells or other leukocytes through secretion of cytokines, proteins, or peptides. Depending on the cytokines produced, $CD4^+$ T-effector cells are subdivided into T helper 1 cells (Th1), secreting IL-2, IL-3, INF-γ, tumor necrosis factor-alpha (TNF-α) and -β or T helper 2 cells (Th2), producing IL-4, IL-5, IL-10, TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF) (7, 47). Th1-type effector cells are essential for $CD8^+$ T cells because $CD8^+$ T cells require IL-2 cytokine for their proliferation. Importantly, $CD4^+$ T cells were increased in the lungs of rats that received LPT treatment. Thus, LPT application may enhance antitumor activities.

Contrary to $CD4^+$ T cells, $CD8^+$ T cells recognize antigens associated with class I MHC proteins (7, 47). Encounter of tumor antigen specific T cell receptor with antigen/MHC I complex induces $CD8^+$ T cells to proliferate and differentiate. $CD8^+$ effector cells release their lysosomes stuffed with perforin and granzyme through exocytosis. Perforins create a pore into the plasma membrane of tumor cells that allows granzymes to enter the cell and cleave caspases precursors to induce apoptosis (47). If the tumor cell expresses the death receptor, Fas,

interaction of Fas-L with Fas may also induce tumor cell apoptosis (47). LPT produced a considerable increase in $CD8^+$ T cells. This result indicates that LPT may enhance killing of tumor cells by increasing the number of cytotoxic cells. However, at 8 days after tumor injection, it is likely that the transition from innate immunity to adaptive immunity is being observed based on the low number of T cells present at this time point.

Taken together, these data highlights the roles of both innate and adaptive immunity components in the elimination of cancer. The evident contribution of both innate and adaptive immune cells in this study is consistent with numerous studies which demonstrate the important immunosurveillance network between innate and adaptive immune cells (4, 5, 21, 38, 39, 47). An important criterion is the possibility that at 8 days post tumor inoculation, the initial onset of adaptive immunity is being observed.

Gut-associated lymphoid tissue (GALT) derived lymphocytes have been shown to migrate to the lungs in response to infection or inflammation (45), reflecting the preferential migration of mucosal lymphocytes stimulated to mucosal sites. Therefore, it was important to investigate the source of leukocytes increased by LPT in order to verify if application of LPT treatment mobilizes gastrointestinal mucosa derived lymphocytes in our rat model consistent to ongoing dog studies. The presence of integrin α4β7-bearing cells was anticipated if a mucosal tissue, such as the GALT, was the source of lung leukocytes. In fact, gastrointestinal lymphocyte trafficking into the lungs of LPT rats was approximately 1.5 times higher compared to control and sham. Although the number of integrin α4β7-bearing cells in the lungs do not account for the total lung leukocytes increased by LPT, this study demonstrates that GALT is a source of the leukocytes increased by LPT.

The effects of interferon γ (IFN-γ) on the immune system against cancer are significant. IFN-γ activates macrophages, increases the expression of class I and class II MHC molecules, and augments NK-cell activity (7). Increased expression of class I MHC increases the display of tumor antigen to $CD8^+$ cells, making antigen-presenting cells more effective at inducing cytotoxic T-cell populations. In addition, an up-regulation of class II MHC molecules on such antigen-presenting cells makes them better presenters of antigen to T_H cells. Thus, *in vivo* cytokine ELISA was performed to determine the concentration of IFN-γ cytokine in the serum following 8 days of intravenous tumor inoculation.

The slight increase in serum IFN-γ from LPT rats represents the enhanced immune response against tumors. However, based on the 8 day time point at which rats were euthanized, it may be possible that higher concentrations of IFN-γ were produced earlier in the time course of our study.

Several studies demonstrate that NK cells play a significant role in the early control of MADB106 (36, 39) but there is also evidence on the influence of B cells in the antitumor production of IFN-γ (38). Therefore, at 8 days post tumor inoculation, the immune response against MADB106 may be transitioning from innate immunity to adaptive immunity. In addition, *in vitro* studies were conducted to determine the impact of LPT treatment on splenocyte IFN-γ production. It was demonstrated that LPT caused no increase in the production of IFN-γ cytokine, indicating that application of lymphatic pump treatment may not affect immune cell function. Additionally, the similar concentration of IFN-γ in spleen culture supernatants of control, sham, and LPT rats upon tumor stimulation or stimulation with culture media alone suggest that adaptive immune cells may not be involved in antitumor effector functions at 8 days after MADB106 injection.

The major function of IL-2, one of the proinflammatory cytokines produced by T cells, is to enhance the growth and cytotoxic response of activated T cells (47). Since IL-2 has been shown to enhance cellular immune responses against tumors by stimulating the proliferation and activation of several types of leukocytes with antitumor activity, including NK cells, antigen-specific T helper cells, cytotoxic lymphocytes, macrophages, and B cells, the *in vitro* production of this cytokine upon co-culture of MADB106 with splenocytes was analyzed. Surprisingly, spleen lymphocytes of control, sham, or LPT animals produced almost undetectable amounts of IL-2 when incubated with or without tumor cells. One possible explanation for this result may be the ability of tumor cells to avoid the immune system by modulating effective responses against tumors and enhancing less effective ones (47). For example, tumors may be able to enhance Th2 responses versus Th1 responses in order to contribute to their survival (47). Other possibility may be that antitumor activity at this point is derived from innate immune cells, thus no IL-2 cytokine is been produced in response to tumors. Furthermore, the lower *in vitro* production of IL-2 in sham and LPT groups may represent the inhibitory effect of daily administration of propofol anesthesia or handling stress on T lymphocyte proliferation.

Although results from our *in vitro* spleen cytokine assays suggest immune response activities against lung cancer, it is likely for IFN-γ and IL-2 *in vitro* cytokine production of lung leukocytes to be different since lung is the metastatic tissue of MADB106. Measurement of *in vitro* lung leukocyte cytokine production would closely represent the local immune response of leukocytes upon stimulation with tumor. Moreover, IL-2 production of regional lymph node leukocytes may highly differ to spleen leukocytes since lymph nodes are a major site of T cell proliferation.

The mechanism responsible for the propofol anesthesia inhibitory effect on sham and LPT animal groups to gain weight during the course of the study is unclear. Nevertheless, several studies correlate the use of propofol as a general anesthetic with different effects such as increased corticosterone secretion during recovery period, induction of anterograde and retrograde amnesia, and disturbance of rest-activity and core body temperature circadian rhythms (48, 49, 50). In rodents, corticosterone is a steroid hormone involved in the regulation of fuel, immune reactions and stress responses. Therefore, it is possible that in our model propofol may be affecting fuel regulation, learning and memory processes, or that its sedative effects may be decreasing food-intake. In addition, the impact on circadian rhythms of rest-activity and body temperature may be responsible for potential symptoms such as fatigue, nausea, drowsiness, or cognitive impairments on postaneshtesia days. On the other hand, it is also possible that sham and LPT animals experience higher levels of handling stress compared to control which may inhibit an increase in body weight.

A limitation to these studies is the duration of the study itself. Application of control, sham, or LPT treatment from day 1-7 and tissue harvest on day 8 may limit the observation of anti-MADB106 immune responses to non-specific innate immunity. However, results collected from these studies provide promising data to focus on the mechanisms involved in the reduction of pulmonary tumors in LPT rats and the effect of LPT on tumor metastasis following the induction of primary tumors by subcutaneous injection.

Conclusion

Collectively, results from this study suggest that application of LPT reduces solid tumors in the lungs, enhances leukocyte trafficking into lungs burden with tumors, and it does not cause

dissemination of tumors outside of the lungs which is the metastatic tissue for MADB106. Therefore, these results provide experimental evidence for the safe use of LPT as a valuable cancer therapy. In addition, our results indicate that GALT is a source of lymphocytes which are mobilized into the lungs during LPT to inhibit tumor establishment. However, whether leukocytes trafficking into the lungs posses enhanced anti-tumor activities to clear pulmonary tumors is still to be determined.

Future directions

In future experiments, the mechanisms responsible for the reduction of pulmonary tumors upon application of lymphatic pump technique (LPT) need to be investigated. To address this question, leukocytes will be isolated and analyzed for their anti-tumor activities. Specifically, the lytic activity of NK cells against MADB106 tumor cells will be evaluated by *in vitro* NK cell tumor lysis assays. NK cell cytolytic activity will be measured by labeling NK target MADB106 tumor cells with PKH-26 cellular membrane dye. The NK-mediated lysis of MADB106 will be determined by incubation of lung and spleen NK cells with PKH-26 labeled tumor target cells at selected effector: target-cell ratios. Next, propidium iodide (PI), which is used to evaluate cell viability, will be added for flow cytometry analysis. NK tumor lysis will be determined based on the percentage of PKH-$26^{+}PI^{+}$ staining. Moreover, it is important to examine the effects of LPT treatment application on early innate immune responses as well as adaptive immune responses against MADB106. Furthermore, since intravenous injection of tumor cells is not representative of true metastasis, the effect of LPT on tumor metastasis upon development of primary tumors by subcutaneous injection will be studied. Finally, further research is necessary to eliminate the possibility that class-I major histocompatibility complex (MHC) on tumor cells has undergone mutations that could inhibit MHC antigen recognition by T cells.

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